



TITLE:

Characterization of the linoleic acid  $\Delta 9$  hydratase catalyzing the first step of polyunsaturated fatty acid saturation metabolism in *Lactobacillus plantarum* AKU 1009a.

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CITATION:

Takeuchi, Michiki ...[et al]. Characterization of the linoleic acid  $\Delta 9$  hydratase catalyzing the first step of polyunsaturated fatty acid saturation metabolism in *Lactobacillus plantarum* AKU 1009a.. *Journal of bioscience and bioengineering* 2014, ...

ISSUE DATE:

2014-12-01

URL:

<http://hdl.handle.net/2433/198741>

RIGHT:

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Characterization of the linoleic acid  $\Delta 9$  hydratase catalyzing the first step of  
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1009a

Running title: Linoleic acid  $\Delta 9$  hydratase from *L. plantarum*

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**Key words:** Lactic acid bacteria; Hydroxy fatty acid; Hydratase; Hydration;  
Dehydration

## 20 ABSTRACT

21 Linoleic acid  $\Delta 9$  hydratase, which is involved in linoleic acid saturation metabolism  
22 of *Lactobacillus plantarum* AKU 1009a, was cloned, expressed as a his-tagged  
23 recombinant enzyme, purified with an affinity column, and characterized. The enzyme  
24 required FAD as a co-factor and its activity was enhanced by NADH. The maximal  
25 activities for the hydration of linoleic acid and for the dehydration of  
26 10-hydroxy-*cis*-12-octadecenoic acid (HYA) were observed at 37°C in buffer at pH 5.5  
27 containing 0.5 M NaCl. Free C16 and C18 fatty acids with *cis*-9 double bonds and  
28 10-hydroxy fatty acids served as substrates for the hydration and dehydration reactions,  
29 respectively. The apparent  $K_m$  value for linoleic acid was estimated to be 92  $\mu\text{M}$ , with a  
30  $k_{cat}$  of  $2.6 \cdot 10^{-2} \text{ sec}^{-1}$  and a Hill factor of 3.3. The apparent  $K_m$  value for HYA was  
31 estimated to be 98  $\mu\text{M}$ , with a  $k_{cat}$  of  $1.2 \cdot 10^{-3} \text{ sec}^{-1}$ .

## 33 INTRODUCTION

34 Hydroxy fatty acids are derived from a variety of natural sources, including  
35 microorganisms, plants, animals, and insects; they are found in triacylglycerols, waxes,  
36 cerebrosides, and other lipids. Hydroxy fatty acids are versatile starting materials that  
37 have been utilized to produce resins, waxes, nylons, plastics, lubricants, biopolymers,  
38 and biodiesel (1). They also have useful antibiotic, anti-inflammatory, and anticancer  
39 activities (2). For example, ricinoleic acid (12-hydroxy-*cis*-9-octadecenoic acid),  
40 derived from castor oil, is converted to sebacic acid (decanedioic acid), the monomer  
41 for nylon synthesis, and has anti-inflammatory and antinociceptive activity (3).

42 Many microorganisms can convert oleic acid (*cis*-9-octadecenoic acid) into  
43 10-hydroxyoctadecanoic acid (1). It has been recently reported that the

myosin-crossreactive antigens (MCRA) from *Elizabethkingia meningoseptica* (4), *Streptococcus pyogenes* (5), and *Bifidobacterium breve* (6) can also convert oleic acid into 10-hydroxyoctadecanoic acid. The hydration of unsaturated fatty acids has been suggested to be a detoxification mechanism in bacteria harboring MCRA proteins and a survival strategy for living in fatty acid-rich environments (5), indicating that MCRA proteins are fatty acid hydratases. A few of the microorganisms having an MCRA protein, including *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Streptococcus bovis*, *Nocradia cholesterolicum*, *Pediococcus pentosaceus*, and *Pediococcus* sp. can convert linoleic acid (LA, *cis*-9,*cis*-12-octadecadienoic acid) to 10-hydroxy-*cis*-12-octadecenoic acid (HYA), 13-hydroxy-*cis*-9-octadecenoic acid, or 10,13-dihydroxyoctadecanoic acid (7–12).

We found that HYA is the initial intermediate in the biosynthesis of conjugated linoleic acid (CLA) from LA in *L. acidophilus* (8). CLAs, which are isomers of LA, have beneficial effects, such as preventing tumorigenesis (13) and arteriosclerosis (14) and decreasing body fat content (15). In our previous study, we screened lactic acid bacteria for the ability to produce CLA from LA, and selected *Lactobacillus plantarum* AKU 1009a as a potential strain (16–18). *L. plantarum* AKU 1009a can transform not only LA but also  $\alpha$ -linolenic acid (*cis*-9,*cis*-12,*cis*-15-octadecatrienoic acid) and  $\gamma$ -linolenic acid (*cis*-6,*cis*-9,*cis*-12-octadecatrienoic acid) into the corresponding conjugated fatty acids (19–24). We also showed that CLA is synthesized, in part, through the reactions of a newly discovered polyunsaturated fatty acid saturation metabolism in *L. plantarum* AKU1009a (25, 26). The novel saturation metabolism consisted of four enzymes: CLA-HY (hydratase/dehydratase), CLA-DH (dehydrogenase), CLA-DC (isomerase), and CLA-ER (enonereductase) (25, 26).

68 CLA-HY, CLA-DH, and CLA-DC are responsible for CLA synthesis from LA (25) and  
69 CLA-ER is a key enzyme for the saturation metabolism (26).

70 In this study, we describe the enzymatic and physiochemical characteristics of  
71 CLA-HY, which catalyzes the initial step of the saturation metabolism: the hydration of  
72 LA and the dehydration of HYA. The enzyme was found to be a unique  
73 hydratase/dehydratase demonstrating activity in the presence of FAD and NADH.

## 75 MATERIALS AND METHODS

### 77 Chemicals

78 HYA was prepared as previously described (8, 17). LA and fatty acid-free  
79 (<0.02%) bovine serum albumin (BSA) were purchased from Sigma (St. Louis, USA).  
80 All of the other chemicals were analytical grade and obtained commercially.

### 82 Cloning and expression of recombinant proteins in *E. coli*

83 Primers were designed to amplify the CLA-HY sequence, without a stop codon,  
84 from *L. plantarum* AKU 1009a genomic DNA. The PCR-amplified product was ligated  
85 into expression vector pET101/D-TOPO (Invitrogen, CA, USA), according to the  
86 manufacturer's instruction. The integrity of the cloned gene was verified by DNA  
87 sequencing using a Beckman-Coulter CEQ8000 (Beckman-Coulter, Fullerton, CA,  
88 USA). The resulting plasmid was purified, and then used to transform *E. coli*  
89 Rosetta<sup>TM</sup>2 (DE3) (Novagen, WI, USA). The transformed cells were cultured in  
90 Luria-Bertani (LB) medium at 37°C for 2 h with shaking at 100 rpm, and then  
91 isopropyl-β-thiogalactopyranoside (IPTG) was added to a final concentration of 1.0 mM.

After adding IPTG, the transformed cells were cultivated at 20°C for 8 h with shaking at 100 rpm.

## Preparation of CLA-HY

All purification procedures were performed at 4°C. The transformed cells (8 g) from 1.5 L of culture broth were harvested, suspended in binding buffer (16 mL), and treated 4 times, for 5 min each, with an ultrasonic oscillator (Insinator 201 M; Kubota, Japan). The binding buffer contained 50 mM imidazole in 20 mM potassium phosphate buffer (KPB) (pH 7.4). The cell debris was removed by centrifugation at  $1700 \times g$  for 10 min. The resulting supernatants were used as cell-free extracts. The cell-free extracts were fractioned by ultracentrifugation at  $100,000 \times g$  for 60 min and the supernatant was obtained. The enzyme was purified from the supernatant using a fast protein liquid chromatography (FPLC) system (Amercham Pharmacia Biotech Co., Uppsala, Sweden) equipped with a His Trap HP column (GE Healthcare, Buckinghamshire, England). The column was equilibrated with the binding buffer and the fractions containing CLA-HY were eluted with elution buffer containing 250 mM imidazole in 20 mM KPB (pH 7.4). The fractions containing CLA-HY were collected and dialyzed against 20 mM KPB (pH 6.5).

## Determination of the molecular mass of His-tagged CLA-HY

In order to determine the native molecular mass of His-tagged CLA-HY, the enzyme solution was subjected to high performance gel-permeation chromatography on a G-3000SW column ( $0.75 \times 60$  cm, Tosoh, Tokyo, Japan) at room temperature. It was eluted with 100 mM KPB (pH 6.5) containing 100 mM  $\text{Na}_2\text{SO}_4$  at the flow rate of 0.5

mL/min. The absorbance of the effluent was monitored at 280 nm. The molecular mass of the enzymes was determined from its mobility relative to those of standard proteins.

### Reaction conditions

All operations were performed in an anaerobic chamber. The standard reaction conditions were as follows. The reactions were performed in test tubes (16.5 × 125 mm) that contained 1 mL of reaction mixture (20 mM sodium succinate buffer, pH 5.5) with 0.5% (w/v) LA or 0.1% (w/v) HYA complexed with BSA [0.1% (w/v) or 0.02% (w/v), respectively] as the substrate, 5 mM NADH, 0.1 mM FAD, and 40 μg (= 0.04 U)/mL CLA-HY. One unit was defined as the amount of enzyme that catalyzes the conversion of 1 μmol of LA per min. The reactions were performed under anaerobic conditions in a sealed chamber containing an O<sub>2</sub>-absorbent (Aneropack “Kenki,” Mitsubishi Gas Chemical Co., Ltd., Tokyo, Japan). The reaction mixture was gently shaken (120 strokes/min) at 37°C for 30 min (for hydration) or 15 min (for dehydration). All experiments were performed in triplicates, and the averages of three separate experiments that were reproducible within ±10% are presented in the figures and tables.

### Enzyme activity assay

Reactions were performed under the standard reaction conditions with some modifications, as described below. The effects of cofactors were examined using a reaction mixture containing LA or HYA as the substrate and various cofactors, such as 0.1 mM FMN, 0.1 mM FAD, 5 mM NADH, 5 mM NADPH, 5 mM NAD<sup>+</sup>, and 5 mM NADP<sup>+</sup>, in various combinations. The effect of oxygen was examined by comparing the reactions under anaerobic condition and aerobic condition. The optimal reaction

temperature was examined by incubating the reaction mixture (20 mM sodium succinate buffer, pH 5.5) at various temperatures for 30 min (for hydration) or 15 min (for dehydration) under anaerobic conditions. The optimal reaction pH was determined at 37°C using 20 mM sodium succinate buffer (pH 4.5–6.0) and 20 mM KPB (pH 5.5–7.0). The effect of NaCl concentration was examined by measuring the enzyme activity of reaction mixtures containing NaCl (0–1 M). Thermal stability was determined by measuring the enzyme activity after incubating reaction mixtures containing 20 mM sodium succinate buffer (pH 5.5) and 0.1 mM FAD at various temperatures for 30 min under anaerobic condition. The pH stability was determined by measuring the enzyme activity after incubating at 37°C for 10 min in the following buffers under anaerobic conditions: sodium citrate buffer (50 mM; pH 3.0–4.0), sodium succinate buffer (50 mM; pH 4.0–6.0), KPB (50 mM; pH 5.0–8.0), and Tris-HCl buffer (50 mM; pH 7.0–9.0).

### **Kinetic analysis**

All operations were performed in an anaerobic chamber. Reactions were performed under standard reaction conditions with modified substrate concentrations. The kinetics of LA hydration were studied using 50–400  $\mu$ M LA complexed with 0.1% (w/v) BSA as the substrate and reaction times of 15 min. The kinetics of HYA dehydration were studied using 50–400  $\mu$ M HYA complexed with or 0.02% (w/v) BSA as the substrate and reaction times of 30 min. The kinetic parameters were calculated by fitting the experimental data to the Hill equation or the Michaelis-Menten equation using KaleidaGraph 4.0 (Synergy Software Inc., PA, USA).



## Lipid analysis

Before lipid extraction, *n*-heptadecanoic acid was added to the reaction mixture as an internal standard. Lipids were extracted from 1 mL of the reaction mixture with 5 mL of chloroform/methanol/1.5% (w/v) KCl in H<sub>2</sub>O (2:2:1, by volume) according to the procedure of Bligh-Dyer, and then concentrated by evaporation under reduced pressure (27). The resulting lipids were dissolved in 1 mL of dichloromethane and methylated with 2 mL of 4% methanolic HCl at 50°C for 20 min. After adding 1 mL of water, the resulting fatty acid methyl esters were extracted with 5 mL of *n*-hexane and concentrated by evaporation under reduced pressure. The resulting fatty acid methyl esters were analyzed by gas-liquid chromatography (GC) using a Shimadzu (Kyoto, Japan) GC-1700 gas chromatograph equipped with a flame ionization detector, a split injection system, and a capillary column (SPB-1, 30 m × 0.25 mm I.D., SUPELCO, PA, USA). The initial column temperature, 180°C for 30 min, was subsequently increased to 210°C at a rate of 60°C/min, and then maintained at 210°C for 29.5 min. The injector and detector were operated at 250°C. Helium was used as a carrier gas at a flow rate of 1.4 mL/min. The fatty acid peaks were identified by comparing the retention times to those of known standards.

## Enantiomeric purity analysis of hydroxy fatty acids

The enantiomeric purities of HYA, 10-hydroxy-*cis*-12,*cis*-15-octadecadienoic acid, and 10-hydroxy-*cis*-6,*cis*-12-octadecadienoic acid were analyzed by Mosher's method (28) using a Bruker Avance III 500 (500MHz) NMR.

## RESULTS

## **Purification of CLA-HY**

The relative molecular mass was calculated to be 52 kDa by high performance gel-permeation chromatography on a G-3000SW column. Purified CLA-HY displayed a single band on an SDS-PAGE gel (Fig. 1). Observed molecular weight of purified CLA-HY was 66 kDa, which was corresponding to calculated mass of 68 kDa deduced from the amino acid sequence of its gene. The purified CLA-HY was used for enzymatic characterization.

## **Effects of cofactors and oxygen**

The effects of potential cofactors FMN, FAD, NADH, NADPH, NAD<sup>+</sup>, and NADP<sup>+</sup> were examined (Fig. 2). Hydration and dehydration activity were observed only in the reaction mixtures containing FAD. The addition of NADH or NADPH with FAD increased these activities by a factor of approximately thirty. The effect of oxygen was examined using reaction mixtures containing FAD and NADH under aerobic conditions. The activity under aerobic conditions was 40–50% of that under anaerobic conditions, which were maintained in a sealed chamber with an O<sub>2</sub> absorbent (Fig. 3).

## **Effects of reaction conditions**

The effects of pH on the activity of CLA-HY were examined over the pH range from 4.5 to 7.0 (Fig. 4a). The enzyme showed maximal activity at pH 5.5. The effects of reaction temperature on the activity of CLA-HY were also examined. The optimal temperature was found to be 37–42°C (Fig. 4b). The effects of FAD concentration were examined from 0 to 100 μM FAD, with or without NADH (Fig. 4c). The hydration and dehydration activities were 10 times greater with NADH than that without NADH. The

enzyme activity increased with increasing FAD concentration up to 20  $\mu$ M with NADH and up to 1  $\mu$ M without NADH. The activities remained the same at higher concentrations of FAD, except in the case of hydration without NADH, which showed decreasing activity with concentrations of FAD above 1  $\mu$ M. The effect of NADH concentration was examined from 0 to 5 mM (Fig. 4d). The hydration and dehydration activities increased with increasing NADH concentration. The effects of NaCl concentration was examined from 0 to 1M (Fig. 4e). The enzyme showed its highest activity with 0.5 M NaCl, which was three times higher than that without NaCl.

### Enzyme stability

The thermal stability of the purified enzyme was investigated from 4°C to 42°C. The enzyme was incubated at each temperature with or without FAD (Fig. 5a). The enzyme was more stable with FAD than that without FAD. More than 80% of the initial activity remained at temperatures up to 32°C in the presence of FAD, and at temperatures up to 28°C in the absence of FAD. The pH stability of the purified enzyme was investigated by incubating the enzyme in different buffers within the pH range 3.0 to 9.0. More than 80% of the initial activity remained in the pH range 4.5 to 6.5 (Fig. 5b).

### Substrate specificity

In the hydration reaction, free C16 and C18 fatty acids with a *cis* carbon–carbon double bond at the  $\Delta$ 9 position, such as LA, palmitoleic acid (*cis*-9-hexadecenoic acid), oleic acid,  $\alpha$ -linolenic acid,  $\gamma$ -linolenic acid, stearidonic acid, and ricinoleic acid, served as substrates and were transformed into the corresponding 10-hydroxy fatty acids. In contrast, fatty acids with a *trans* carbon–carbon double bond at  $\Delta$ 9 position (elaidic acid,

*trans*-9-octadecenoic acid), fatty acid esters (methyl linoleate, monolinolein, dilinolein, and trilinolein), and conjugated fatty acids (conjugated linoleic acids) were not hydrated. Fatty acids with other chain lengths, such as myristoleic acid (*cis*-9-tetradecenoic acid), arachidonic acid (*cis*-5,*cis*-8,*cis*-11,*cis*-14-eicosatetraenoic acid), EPA (*cis*-5,*cis*-8,*cis*-11,*cis*-14,*cis*-17-eicosapentaenoic acid), and DHA (*cis*-4,*cis*-7,*cis*-10,*cis*-13,*cis*-16,*cis*-19-docosahexaenoic acid) were not hydrated; nor were fatty acids with a *cis* carbon-carbon double bond at  $\Delta 11$  position, such as *cis*-vaccenic acid and *cis*-11-octadecenoic acid, or fatty alcohols, such as linoleyl alcohol (Table 1).

In the dehydration reaction, 10-hydroxy C18 fatty acids, such as HYA, 10-hydroxy-*cis*-12,*cis*-15-octadecadienoic acid, 10-hydroxy-*cis*-6,*cis*-12-octadecadienoic acid, and 10-hydroxyoctadecanoic acid served as substrates and were transformed into the corresponding fatty acids with *cis* double bonds at the  $\Delta 9$  position. However, 12-hydroxy, 3-hydroxy, and 9-hydroxy fatty acids were not dehydrated (Table 2).

## Kinetic analysis of the CLA-HY catalyzing reactions

The substrate–velocity curve for LA hydration had a sigmoid shape. When fitted with the Hill equation, the apparent  $K_m$  value for LA was estimated to be 92  $\mu\text{M}$  with a  $k_{cat}$  value of  $2.6 \cdot 10^{-2} \text{ sec}^{-1}$  and a Hill factor of 3.3. The apparent  $K_m$  value for HYA in the dehydration reaction was estimated to be 98  $\mu\text{M}$  with a  $k_{cat}$  of  $1.2 \cdot 10^{-3} \text{ sec}^{-1}$ .

## Enantiomeric purities of the produced hydroxy fatty acids

The carbons bearing hydroxy functional groups in the hydroxy fatty acids

produced during the hydration reaction are asymmetric. The enantiomeric purities of the HYA, 10-hydroxy-*cis*-12,*cis*-15-octadecadienoic acid, and 10-hydroxy-*cis*-6,*cis*-12-octadecadienoic acid produced by CLA-HY were analyzed using Mosher's method. They were found to be of the (*S*)-configuration with more than 99.9% enantiomeric excess (*e.e.*).

### Effects of chemicals on the enzyme activity

The effects of monovalent and divalent metal ions (1 mM) were investigated in both the hydration and dehydration reactions. The reactions were strongly inhibited by Ag<sup>+</sup>, Fe<sup>2+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup>, Hg<sup>2+</sup>, and Fe<sup>3+</sup> (data not shown). Dodecanoic acid was reported to form an insoluble complex with metal ions (Cu<sup>2+</sup>, Zn<sup>2+</sup>, Fe<sup>3+</sup>, Co<sup>2+</sup>, and Ni<sup>2+</sup>) (29). One of the reasons these metal ions inhibited the reaction may have been the formation of substrate complexes like those observed with dodecanoic acid. In contrast, a slight increase in the hydration activity was observed with MnCl<sub>2</sub> (1 mM).

The effects of various enzyme inhibitors (1 mM), such as SH-reagents, carbonyl reagents, serine protease inhibitors, and redox indicators, were investigated. Significant inhibition was found only with triphenyl tetrazolium chloride, a redox indicator that can serve as a strong electron acceptor.

## DISCUSSION

Many bacteria have an MCRA protein to detoxify unsaturated fatty acids by transforming them into hydroxy fatty acids. CLA-HY, which belongs to the MCRA family, should also detoxify unsaturated fatty acids in *L. plantarum*, because the growth of *L. plantarum* is inhibited by LA (16).

CLA-HY required FAD for activity; its activity was further increased by the addition of NADH (Fig. 2 and Fig. 4c). These results indicate that CLA-HY is an FAD-dependent enzyme, and NADH is an activator of CLA-HY. Almost all MCRA proteins have an FAD-binding motif, such as a GXGXXS(A/G) (30), but the corresponding sequence in CLA-HY is GAGLSN. FAD might bind loosely to CLA-HY, because the purified CLA-HY, after dialysis, showed no absorbance at 450 nm (data not shown).

The absorbance of FAD at 450 nm was decreased by the addition of NADH, indicating that FADH<sub>2</sub> is the active cofactor and is produced through the reduction of FAD by NADH. Oxygen inactivated the enzyme because FADH<sub>2</sub> is easily oxidized by oxygen (Fig. 3). The mechanism of NADH activation was similar to that described for 2-haloacrylate hydration by 2-haloacrylate hydratase (31). FADH<sub>2</sub> may be involved in the activation of a water molecule that attacks the  $\Delta^9$  double bond of LA, or in the protonation of the C10 carbon of LA. As for dehydration, the hydroxy group of the hydroxy fatty acid may be activated by FADH<sub>2</sub>. In addition to this activation, FADH<sub>2</sub> may also have a role in stabilizing the enzyme through its reducibility.

There are few reports concerning the dehydration of hydroxy fatty acids. In many cases, the substrates are 2-hydroxy or 3-hydroxy fatty acyl-CoAs, which are intermediates in the elongation of fatty acids and are dehydrated by enoyl-CoA hydratase (32). In this paper, we described the enzymatic dehydration of 10-hydroxy fatty acids for the first time. A detailed analysis of the CLA-HY-catalyzed reaction provided novel information about enzymatic dehydration: the requirement for FAD and activation by NADH. Such information may be useful for creating industrially important dehydration catalysts for the synthesis of monomers for radical

polymerization. The possibilities include the synthesis of acrylamide, propylene, and styrene from alcohols 3-hydroxypropionate/lactate, propanol, and ethanol, respectively.

The newly generated double bonds in the products of CLA-HY-catalyzed dehydration were in the *cis*-configuration in this study, whereas the generation of double bonds in the *trans*-configuration was observed in our previous study (26). These results indicate the possibility that some reaction conditions or additional factors affect the geometric selectivity of the dehydration reaction. Because *trans* fatty acids are reported to be harmful for health, the geometric selectivity of the hydration reaction must be controlled to reduce *trans* fatty acids in dietary foods. We are investigating the factors that control geometric selectivity in CLA-HY-catalyzed hydration. The results of the detailed analysis will be presented in future reports.

Hydroxy fatty acids are important materials for the chemical, food, cosmetic, and pharmaceutical industries; they have also attracted recent interest from a variety of research fields. For example, hydroxy fatty acids can be applied to the production of biopolymers, the improvement of health, and the production of pharmaceuticals with anti-inflammatory and antinociceptive effects. Not only HYA and 10-hydroxyoctadecanoic acid but also 10-hydroxy-*cis*-12,*cis*-15-octadecadienoic acid, 10-hydroxy-*cis*-6,*cis*-12-octadecadienoic acid, 10-hydroxy-*cis*-6,*cis*-12,*cis*-15-octadecatrienoic acid, and 10,12-dihydroxyoctadecanoic acid can be produced by CLA-HY-catalyzed reactions. CLA-HY showed regioselectivity for  $\Delta^9$  double bond hydration, generating C10 hydroxy groups in the (*S*)-configuration with high enantioselectivity, while chemical hydration has some difficulties with regio- and stereo-selectivities. These characteristics of CLA-HY enable the fine synthesis of hydroxy fatty acids. These products with fine structures could be

useful for their precise functional evaluation for application purposes.

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## ACKNOWLEDGMENTS

This work was supported, in part, by the Industrial Technology Research Grant Program in 2007 (Grant 07A08005a to S.K.); the Project for the Development of a Technological Infrastructure for Industrial Bioprocesses on Research and Development of New Industrial Science and Technology Frontiers (S.S.) from the New Energy and Industrial Technology Development Organization (NEDO) of Japan; Scientific Research Grants in Aid 19780056 (to S.K.), 16688004 (to J.O.), and 18208009 (to S.S.); the Centers of Excellence for Microbial-Process Development Pioneering Future Production Systems from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; the Bio-Oriented Technology Research Advancement Institution of Japan (J.O.); Advanced Low Carbon Technology Research and Development Program of Japan. M.T. and S.K. received Research Fellowship from the Japan Society for the Promotion of Science for Young Scientists.

## Figure legends

**Fig. 1 SDS-PAGE analysis of purified CLA-HY.** Molecular mass standards: from the top, phosphorylase b (97,200), bovine serum albumin (66,400), ovalbumin (45,000),

carbonic anhydrase (29,000), and trypsin inhibitor (20,100). Observed molecular weight of purified CLA-HY was 66 kDa.

**Fig. 2 Effects of cofactors on the activity of CLA-HY.** Hydration activity (black bars) and dehydration activity (white bars) were assayed under standard reaction conditions except for the addition of cofactors FMN, FAD, NADH, NADPH, NAD<sup>+</sup>, NADP<sup>+</sup> in various combinations.

**Fig. 3 Effect of oxygen on the activity of CLA-HY.** Hydration activity (black bars) and dehydration activity (white bars) were assayed under standard reaction conditions. Aerobic reactions were conducted in an open chamber. Anaerobic reactions were performed in a sealed chamber with O<sub>2</sub>-absorbent.

**Fig. 4 Effects of pH, temperature, and the concentration of FAD, NADH, and NaCl on the activity of CLA-HY.** a, Effects of pH. Activity was assayed under standard reaction conditions, except for the buffers used. Sodium succinate buffer (closed and open circles for hydration and dehydration, respectively), pH 4.5–6.0, and potassium phosphate buffer (closed and open triangles for hydration and dehydration, respectively), pH 5.5–7.0, were used. b, Effects of temperature. Hydration activity (closed circles) and dehydration activity (open circles) were assayed under standard reaction conditions, except for the temperature. c, Effects of FAD concentration. Enzyme activity was assayed under standard reaction conditions, except for the FAD concentration with (closed and open circles for hydration and dehydration, respectively) or without (closed and open triangles for hydration and dehydration, respectively) the addition of NADH. d, Effects of NADH concentration. Hydration activity (closed circles) and dehydration activity (open circles) were assayed under standard reaction conditions, except for the NADH concentration. e, Effects of NaCl concentration. Hydration activity (closed

circles) and dehydration activity (open circles) were assayed under standard reaction conditions, except for the addition of NaCl (0–1.0 M).

**Fig. 5 Effects of temperature and pH on stability of CLA-HY.** a, Effects of temperature. The thermal stability of the hydration activity was assessed under standard reaction conditions after incubation at each temperature (4–42°C) for 30 min with (closed circles) or without (closed triangles) FAD. The thermal stability of the dehydration activity was assessed under standard condition after incubation at each temperature (4–42°C) for 30 min with (open circles) or without (open triangles) FAD. The activities after incubation with FAD at 4°C or at 18°C were defined as 100% for hydration (1.1 U/mg) and dehydration (0.020 U/mg), respectively. b, Effect of pH. The pH stabilities of the hydration (closed) and dehydration (open) reactions were evaluated under standard reaction conditions after incubation at 37°C for 10 min at each pH. Sodium citrate buffer, pH 3.0–4.0 (circles), sodium succinate buffer, pH 4.0–6.0 (triangles), potassium phosphate buffer, pH 5.0–8.0 (diamonds), and Tris-HCl buffer, 7.0–9.0 (squares) were used. The activities after incubation in sodium succinate buffer (pH5.5) and in sodium succinate buffer (pH 6.0) were defined as 100% for the hydration (1.2 U/mg) and dehydration (0.13 U/mg), respectively.

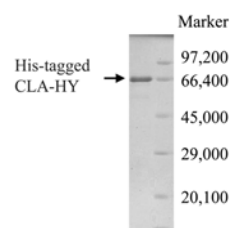


Fig. 1 SDS-PAGE analysis of purified CLA-HY.

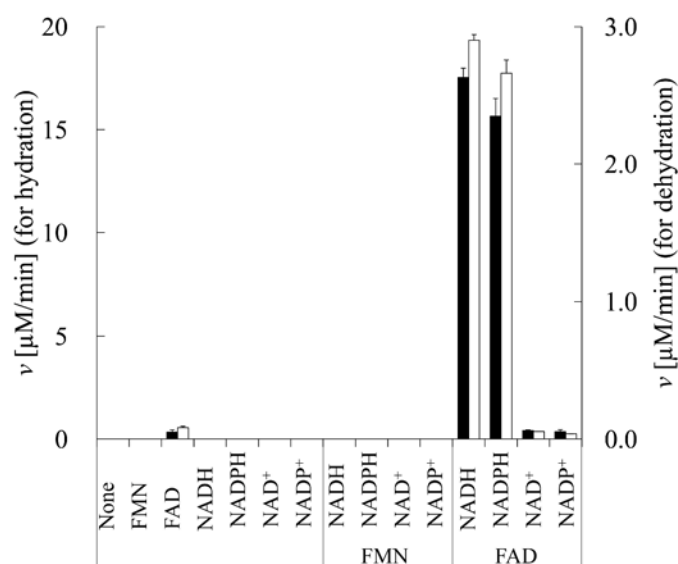


Fig. 2 Effects of cofactors on the activity of CLA-HY.

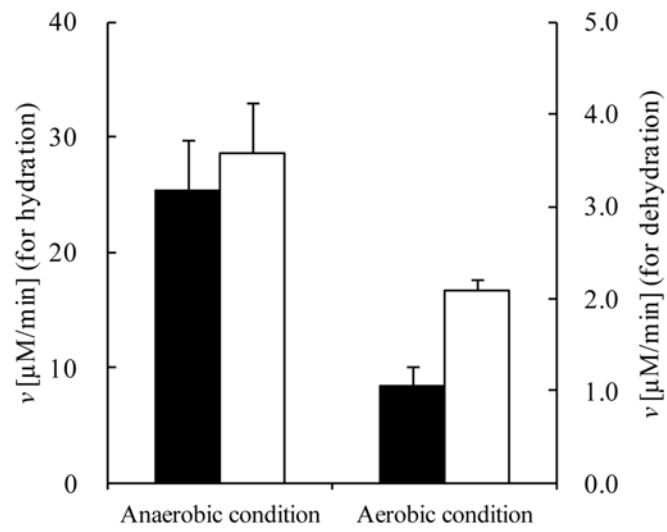


Fig. 3 Effect of oxygen on the activity of CLA-HY.

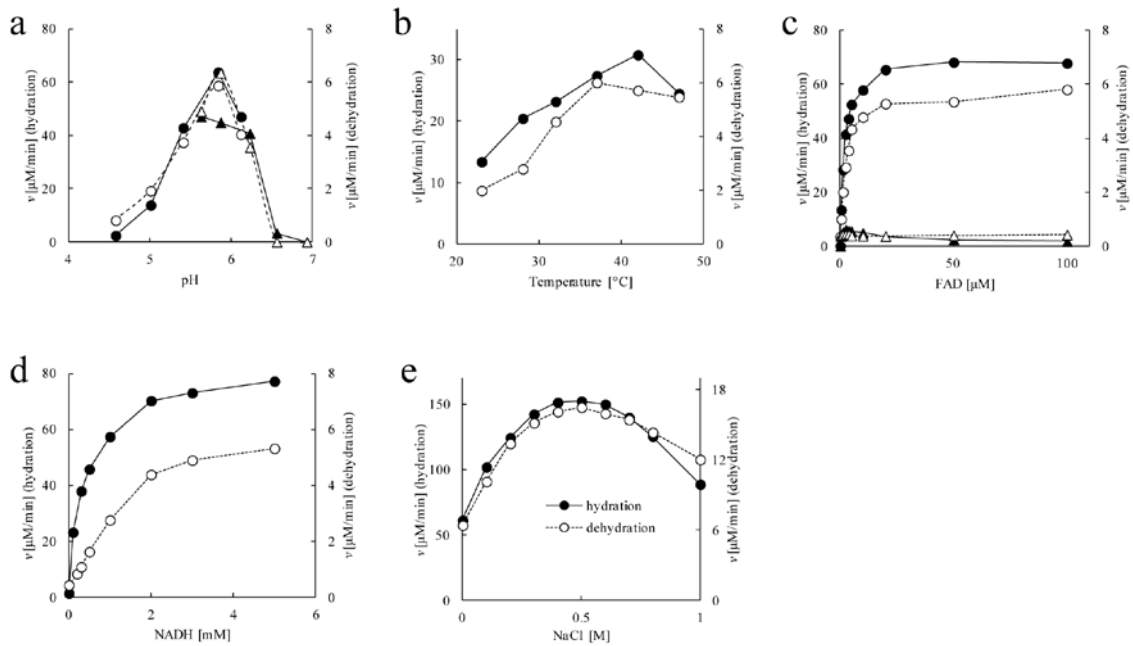


Fig. 4 Effects of pH, temperature, and the concentration of FAD, NADH, and NaCl on the activity of CLA-HY.



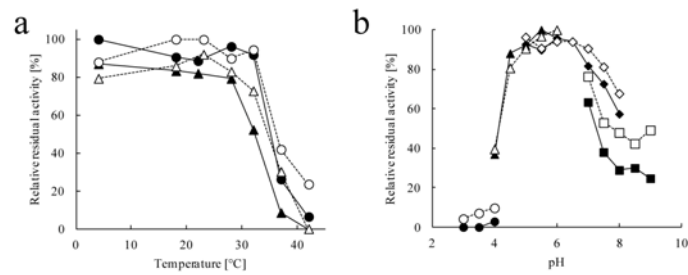


Fig. 5 Effects of temperature and pH on stability of CLA-HY.

**Table 1 Substrate specificity of CLA-HY-catalyzed hydration**

Substrate	Product	Relative activity [%]
<i>cis</i> -9, <i>cis</i> -12-Octadecadienoic acid (Linoleic acid)	10-Hydroxy- <i>cis</i> -12-octadecenoic acid	100 <sup>a</sup>
<i>cis</i> -9-Tetradecenoic acid (C14) (Myristoleic acid)		- <sup>b</sup>
<i>cis</i> -9-Hexadecenoic acid (C16) (Palmitoleic acid)	10-Hydroxyhexadecanoic acid	44
<i>cis</i> -9-Octadecenoic acid (C18) (Oleic acid)	10-Hydroxyoctadecanoic acid	335
<i>trans</i> -9-Octadecenoic acid (Elaidic acid)		-
<i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15-Octadecatrienoic acid ( $\alpha$ -Linolenic acid)	10-Hydroxy- <i>cis</i> -12, <i>cis</i> -15-octadecadienoic acid	29
<i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -12-Octadecatrienoic acid ( $\gamma$ -Linolenic acid)	10-Hydroxy- <i>cis</i> -6, <i>cis</i> -12-octadecadienoic acid	43
<i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15-Octadecatetraenoic acid (Stearidonic acid)	10-Hydroxy- <i>cis</i> -6, <i>cis</i> -12, <i>cis</i> -15-octadecatrienoic acid	43
<i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14-Eicosatetraenoic acid (Arachidonic acid)		-
<i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14, <i>cis</i> -17-Eicosapentaenoic acid (EPA)		-
<i>cis</i> -4, <i>cis</i> -7, <i>cis</i> -10, <i>cis</i> -13, <i>cis</i> -16, <i>cis</i> -19-Docosahexaenoic acid (DHA)		-
<i>cis</i> -9, <i>cis</i> -12-Octadecadienol (Linoleyl alcohol)		-
12-Hydroxy- <i>cis</i> -9-octadecenoic acid (Ricinoleic acid)	10,12-Dihydroxyoctadecanoic acid	0.5
<i>cis</i> -11-Octadecenoic acid ( <i>cis</i> -Vaccenic acid)		-
<i>cis</i> -9, <i>trans</i> -11-Octadecadienoic acid ( <i>cis</i> -9, <i>trans</i> -11-Conjugated linoleic acid)		-
<i>trans</i> -10, <i>cis</i> -12-Octadecadienoic acid ( <i>trans</i> -10, <i>cis</i> -12-Conjugated linoleic acid)		-
Methyl <i>cis</i> -9, <i>cis</i> -12-octadecadienoate (Methyl linoleate)		-
Monolinolein		-
Dilinolein		-
Trilinolein		-

<sup>a</sup>, The activity of linoleic acid hydration (= 1.2 U/mg) under the condition (0.1 mM FAD, 5 mM NADH; 37°C, pH 5.5, 30 min) was defined as 100%. <sup>b</sup>, not detected.

**Table 2 Substrate specificity of CLA-HY-catalyzed dehydration**

Substrate	Product	Relative activity [%]
10-Hydroxy- <i>cis</i> -12-octadecenoic acid	<i>cis</i> -9, <i>cis</i> -12-Octadecadienoic acid (Linoleic acid)	100 <sup>a</sup>
10-Hydroxy- <i>cis</i> -12, <i>cis</i> -16-octadecadienoic acid	<i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15-Octadecatrienoic acid ( $\alpha$ -Linolenic acid)	127
10-Hydroxy- <i>cis</i> -6, <i>cis</i> -12-octadecadienoic acid	<i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -12-Octadecatrienoic acid ( $\gamma$ -Linolenic acid)	61
10-Hydroxyoctadecanoic acid	<i>cis</i> -9-Octadecenoic acid (Oleic acid)	<i>tr.</i> <sup>b</sup>
12-Hydroxy- <i>cis</i> -9-octadecenoic acid		- <sup>c</sup>
12-Hydroxyoctadecanoic acid		-
3-Hydroxyhexadecanoic acid (C16)		-
9-Hydroxynonanoic acid (C9)		-

<sup>a</sup>, The activity of 10-hydroxy-*cis*-12-octadecenoic acid dehydration (= 0.040 U/mg) under the condition (0.1 mM FAD, 5 mM NADH; 37°C, pH 5.5, 15 min) was defined as 100%. <sup>b</sup>*tr.*, trace, <0.001%. <sup>c</sup>, not detected.